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Zooxanthellae of the *Montastraea annularis* Species Complex: Patterns of Distribution of Four Taxa of *Symbiodinium* on Different Reefs and Across Depths

W. W. TOLLER[1,3], R. ROWAN[2,*], AND N. KNOWLTON[1,3]

[1]*Marine Biology Research Division 0202, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093-0202;* [2]*University of Guam Marine Laboratory, Mangilao, Guam 96923; and* [3]*Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama*

Abstract. Corals of the *Montastraea annularis* complex host several different dinoflagellates in the genus *Symbiodinium*. Here we address two questions arising from our previous studies of these associations on an offshore reef. First, do the same taxa and patterns of association (*Symbiodinium* A and B found in higher irradiance habitats than *Symbiodinium* C) occur on an inshore reef? Second, does *M. franksi* at the limits of its depth range host only *Symbiodinium* C, as it does at intermediate depths? In both surveys, a new *Symbiodinium* taxon and different patterns of distribution (assayed by analyses of small ribosomal subunit RNA genes [srDNA]) were observed. Inshore, a taxon we name *Symbiodinium* E predominated in higher irradiance habitats in *M. franksi* and its two sibling species; the only other zooxanthella observed was *Symbiodinium* C. Offshore, *M. franksi* mainly hosted *Symbiodinium* C, but hosted *Symbiodinium* A, B, C, and E in shallow water and *Symbiodinium* E and C in very deep water. *Symbiodinium* E may be stress-tolerant. Observed srDNA heterogeneity within samples of *Symbiodinium* B, C, and E is interpreted as variation across copies within this multigene family. Experimental bleaching of *Symbiodinium* C supported this interpretation. Thus sequences from natural samples should be interpreted cautiously.

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* To whom correspondence should be addressed. E-mail: rrowan@uog9.uog.edu

Abbreviations: RFLP, restriction fragment length polymorphism; rDNA, ribosomal RNA genes; srDNA, small ribosomal subunit RNA genes.

Introduction

Coral reefs are the most biologically diverse marine habitats. Underpinning this diversity are the reef-building corals themselves, which are obligate, mutualistic symbioses between coral animals and dinoflagellates (commonly called zooxanthellae). This partnership between heterotrophic hosts and phototrophic symbionts allows corals to thrive in shallow, nutrient-poor tropical seas, and deposit calcium carbonate in amounts large enough to build reefs (reviewed in Muscatine and Porter, 1977; Falkowski *et al.*, 1984; Barnes and Chalker, 1990; Muller-Parker and D'Elia, 1997).

Coral taxonomy at the species level, although occasionally frustrating (Knowlton and Jackson, 1994; Veron, 1995), has generally been sufficient to describe overall diversity and to define experimental subjects. This taxonomy seldom, however, has considered zooxanthellae, because it was widely assumed that one species of coral associates with only one species of zooxanthella—in other words, that host taxonomy identified both partners. Zooxanthellae are diverse (*e.g.*, Schoenberg and Trench, 1980; Rowan, 1998), and it is now recognized that some species of corals associate with multiple species of zooxanthellae (Rowan and Knowlton, 1995; Rowan, 1998). Thus corals identified as members of the same species may not in fact be equivalent at the whole organism (holobiont) level, and the taxonomic identities of zooxanthellae may be as ecologically important as those of their hosts.

As far as is known, zooxanthellae in reef-building corals are members of the genus *Symbiodinium* (Rowan, 1998), which includes four species described as *in vitro* cultures (Freudenthal, 1962; Trench and Blank, 1987). Several other

cultured isolates of *Symbiodinium* have been named informally, but most members of the genus remain uncultured and undescribed (Rowan, 1998). Nevertheless, sequences and restriction fragment length polymorphism (RFLP) of genes that encode ribosomal RNA (rDNA) can be used to distinguish some taxa of *Symbiodinium* and to study ecological relationships among host, symbiont, and habitat diversity (Rowan and Powers, 1991a, b; Rowan and Knowlton, 1995; Rowan *et al.*, 1997; Baker and Rowan, 1997; Hill and Wilcox, 1998; Darius *et al.*, 1998; Baker, 1999). The present study uses genes that encode small ribosomal subunit RNA (srDNA).

Our earlier work concerned zooxanthellae of the sibling coral species *Montastraea annularis, M. faveolata,* and *M. franksi,* which are the dominant reef-building corals in the Western Atlantic (Goreau, 1959). On an offshore reef in the San Blas Islands of Panama, we found that both *M. annularis* and *M. faveolata* associate with three distinct taxa of *Symbiodinium* (A, B, and C; see Rowan and Knowlton, 1995; Rowan *et al.*, 1997). *Symbiodinium* A and B, or both, are predominant in tissue exposed to high irradiance (shallower water or colony tops), *Symbiodinium* C is predominant in shaded tissue (deeper water or colony sides), and mixtures of *Symbiodinium* A and/or B with C occur between these extremes. Colonies of *M. franksi,* in contrast, were found to host only *Symbiodinium* C (Rowan and Knowlton, 1995); however, this coral species was not found at shallow depths on this reef. These observations led to two questions addressed here. First, do these symbiont taxa and patterns of association occur on other types of reef? Second, does the deeper distribution of *M. franksi* reflect an inability by this species to host those taxa of *Symbiodinium* with which *M. annularis* and *M. faveolata* associate in shallow water?

We also discuss some concerns about using srDNA to identify the *Symbiodinium* that we collected. Although srDNA was heterogeneous in samples of *Symbiodinium* B, C, and E, we found no evidence to suggest that the zooxanthellae in each of these samples were heterogeneous. We suspect that srDNA in these *Symbiodinium* is a heterogeneous multigene family, as is rDNA in some other dinoflagellates (Scholin *et al.*, 1993; Scholin and Anderson, 1994, 1996). We discuss practical implications of this suspicion for the use of srDNA as a taxonomic character.

Materials and Methods

Field collections and study sites

Corals were identified in the field by colony-level characters (Weil and Knowlton, 1994). Apparently healthy colonies, separated from one another by >2 m, were sampled with hammer and ½-in (#12) steel hole punch, yielding a coral core with about 1.3 cm^2 of live colony surface. Cores were wrapped in aluminum foil and frozen in a cryogenic dry shipper (chilled with liquid nitrogen). Many colonies of

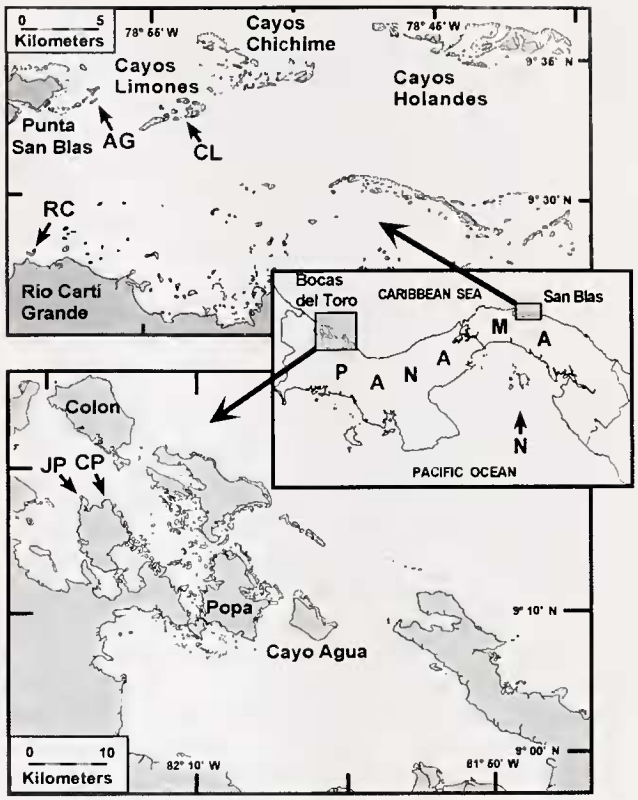

Figure 1. Collecting localities in the San Blas Archipelago (upper panel) and Bocas del Toro (lower panel), Republic of Panama (inset). Arrows with initials identify places where corals were sampled: AG, Aguadargana reef; RC, Río Cartí; CL, Cayos Limones; JP, Juan Point; CP, Cocos Point. Data from Aguadargana reef were reported previously (Rowan and Knowlton, 1995; Rowan *et al.*, 1997).

Montastraea annularis and *M. faveolata* were sampled both on their tops and on their sides to obtain samples from relatively high- and low-irradiance tissues (respectively) within a colony (Rowan *et al.*, 1997). Most colonies of *M. franksi* were sampled at only one location because their relatively flat morphologies made a distinction between colony top and side superfluous.

Coral colonies were sampled at three sites in the Republic of Panama (Fig. 1) between October 1997 and October 1998:

(1) Río Cartí, San Blas. We sampled from a small coastal fringing reef adjacent to the mouth of a major river (Río Cartí Grande). During May to December, such nearshore sites are periodically subjected to heavy freshwater runoff and riverine sediments (Clifton *et al.*, 1997; D'Croz *et al.*, 1999). *Montastraea* species occur at Río Cartí from the barely subtidal to a depth of about 12 m, where hard substrate is replaced by soft-bottom sediments. We sampled the tops of all encountered colonies (*M. annularis*, $n = 4$; *M. faveolata*, $n = 20$; *M. franksi*, $n = 19$); 30 of these were also sampled on their sides.

(2) Cayos Limones, San Blas. These reefs are located 15

km north of mainland Panama and are not strongly influenced by terrestrial runoff (D'Croz *et al.*, 1999). We sampled from a relatively steep, leeward fringing reef that ends abruptly at depths between 35 and 40 m in soft sediments (see fig. 9 in Robertson and Glynn, 1977). On this reef, *M. franksi* is common below 8 m, and it is the dominant coral (with *Agaricia lamarcki*) below 15 m. We sampled *M. franksi* throughout its depth range (4 to 38 m, $n = 78$ colony tops).

(3) Bocas del Toro. Juan Point and Cocos Point reefs are located in the semienclosed lagoon of Bahía Almirante in the Province of Bocas del Toro. Like Río Cartí, these sites are affected by high rainfall and river outflow throughout much of the year. On many of the reefs in this area, *M. franksi* is the most abundant member of the *M. annularis* complex. We made a limited collection at depths of 1-15 m for comparative purposes, consisting of 1 top sample of *M. annularis*, 10 of *M. franksi*, and 3 of *M. faveolata*.

Observations of srDNA heterogeneity within samples of *Symbiodinium* C (see Results) prompted us to investigate the stability of these genotypes under stress. We identified 11 colonies of *M. annularis* (each colony consisting of a cluster of columns) that hosted heterogeneous RFLP genotypes of *Symbiodinium* C. After an initial sample, columns (one per colony) were transplanted from their natural habitat (*ca.* 10-14 m depth) to 1 m depth at either Cayos Limones ($n = 4$ transplants) or Aguadargana ($n = 7$ transplants) reefs (Fig. 1), where they bleached. Columns were sampled again after 4 days (Cayos Limones) or 40 days (Aguadargana). Transplants and determinations of zooxanthellar numbers were conducted as described in Toller *et al.* (2001). In the present study, however, we did not sample corals further (*i.e.*, during zooxanthellar repopulation; see Toller *et al.*, 2001).

Identification of zooxanthellae

Zooxanthellae were isolated and identified as described previously (Rowan and Powers, 1991b; Rowan and Knowlton, 1995). srDNAs were obtained by PCR amplification with a "host-excluding" primer pair (ss5 and ss3Z) or with universal primers (ss5 and ss3), and then characterized by restriction enzyme digestion. The host-excluding primer pair does not amplify known host srDNAs (Rowan and Powers, 1991b; unpubl. obs.), but does amplify srDNAs from a phyletic group that is much larger than *Symbiodinium* (McNally *et al.*, 1994; Toller *et al.*, 2001). All samples were assayed using host-excluding primers, and about one-third of them were also analyzed with universal primers. Data obtained from the two kinds of amplifications were always in agreement.

Every sample was analyzed by digesting amplified srDNA with *Dpn* II and with *Taq* I, which differentiate *Symbiodinium* A, B, and C by RFLP (Rowan and Powers, 1991a; Rowan and Knowlton, 1995; Rowan *et al.*, 1997). RFLPs were diagnosed by comparison to genotype standards, which were obtained by PCR amplification from cloned srDNAs of *Symbiodinium* A, B, and C, all isolated from *M. annularis* (Rowan and Knowlton, 1995), and from *Symbiodinium* E (from *M. faveolata*, this study). These cloned genotype standards are denoted hereafter as A^0, B^0, C^0, and $E^{0\text{-}1}$. We use the superscript zero to indicate srDNA clones, as opposed to taxa of *Symbiodinium*; clones obtained from different samples of the same taxon of *Symbiodinium* are distinguished by numbers (*e.g.*, $E^{0\text{-}1}$ and $E^{0\text{-}2}$; see below). Because universal PCR primers amplify coral host srDNA when it is present (Rowan and Powers, 1991b), a cloned srDNA from *M. annularis* (clone H^0; see below) was used as an additional standard in RFLP analyses of these amplifications. Where RFLP analyses indicated mixtures of *Symbiodinium* A, B, C, or E in a sample, relative abundance (greater than or less than 50% of the total) was estimated by comparison to standard mixtures prepared from cloned srDNAs (Rowan and Knowlton, 1995; Rowan *et al.*, 1997; see Fig. 4).

srDNA was cloned from three samples of *Symbiodinium* E: clone $E^{0\text{-}1}$ is from *M. faveolata* (from Río Cartí, 3 m depth), clone $E^{0\text{-}2}$ is from *M. franksi* (from Cayos Limones, 38 m), and clone $E^{0\text{-}3}$ is from the coral *Siderastrea siderea* (from Portobelo, Panama, 6 m). Amplified srDNAs (DNA for clone $E^{0\text{-}1}$ by universal PCR primers; DNAs for clones $E^{0\text{-}2}$ and $E^{0\text{-}3}$ by host-excluding PCR primers) were gel-purified, ligated into pGEM-T Easy Vector (Promega Corporation, Madison, WI), and then transformed into *Escherichia coli* according to manufacturer's recommendations. From each ligation, 4-12 clones were characterized by amplifying srDNAs with host-excluding PCR primers and then digesting the PCR products with *Dpn* II, with *Taq* I, and with *Hae* III. Each cloned RFLP genotype was compared to the RFLP of its corresponding natural sample. srDNA of *M. annularis* was obtained with universal PCR primers from sperm DNA (Lopez *et al.*, 1999) and cloned (clone H^0) as described above.

Clones $E^{0\text{-}1}$, $E^{0\text{-}2}$, and $E^{0\text{-}3}$ were sequenced completely, as were cloned genotype standards A^0, B^0, and C^0 (from which only partial sequences had been obtained previously; Rowan and Knowlton, 1995) and clone H^0. Plasmids were prepared using QIAprep Spin Miniprep kits (Qiagen, Inc., Valencia, CA) according to manufacturer's recommendations, and sequences were determined for both DNA strands using Big Dye Terminator sequencing kits (PE Corporation, Norwalk, CT) with vector sequencing primers T7 and M13-Reverse, and with srDNA sequencing primers 18F1 (5′-AGCTCGTAGTTGGATTTCTG-3′), 18F2 (5′-TTAATTTGACTCAACACGGG-3′), 18R1 (5′-AGTCAAATTAAGCCGCAGGC-3′) or 18-R1X (5′-GTTGAGTCAAATTAAGCCGC-3′), and 18R2 (5′-ATATACGCTATTGGAGCTGG-3′). Reactions were analyzed with an ABI

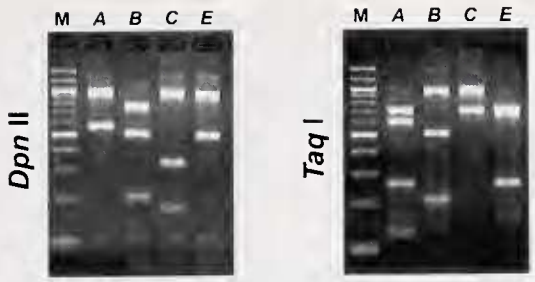

Figure 2. RFLP genotypes A, B, C, and E of *Symbiodinium* obtained from different colonies of *Montastraea franksi*. srDNAs were amplified with host-excluding PCR primers and digested with *Dpn* II (left) and with *Taq* I (right). Lane M contains DNA fragment size standards of (top to bottom) 1500 base pairs (bp), 1200 bp, and then 1000 bp to 100 bp in 100-bp increments.

373 sequencer (Applied Biosystems, Foster City, CA) and complete sequences were assembled using SeqEd software (Applied Biosystems). RFLP genotypes of cloned srDNAs were obtained from their sequences using Gene Construction Kit software (Textco, Inc., West Lebanon, NH). Note that we used only partial srDNA sequences in some analyses (Fig. 3); the full-length srDNA sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/; accession numbers AF238256-AF238258, AF238261-AF238263, and AF238267).

For phylogenetic analysis, we aligned partial srDNA sequences (Rowan and Powers, 1992) with Clustal X software (Thompson *et al.*, 1997) and used neighbor-joining reconstruction (Saitou and Nei, 1987). The following srDNA sequences were obtained from GenBank: *Symbiodinium microadriaticum* (M88521), *Symbiodinium* #8 (M88509), *Symbiodinium* sp. PSP1-05 (AB016578), s11-2xba (U20961), s20-2xba (U20962), 37-4xba (U20959), 86-5xba (U20960), a12-5xba (U20954), a8-5xba (U20955), 175-5xba (U20952), 178-6xba (U20956), 33-6xba (U20958), a1-5xba (U20953), 178-8xba (U20957), *Gymnodinium beii* (U37366), *Gyrodinium galatheanum* (M88511), *Gymnodinium simplex* (M88512), and *Polarella glacialis* (AF099183). srDNA sequences from *Symbiodinium* C2 [clone $C2^{0-1}$ (AF238259) and clone $C2^{0-2}$ (AF238260)] are from Toller *et al.* (2001). A partial sequence of zooxanthellar srDNA from the coral *Montipora patula* is from a previous study (Rowan and Powers, 1991a).

To investigate srDNA variation within our samples of *Symbiodinium* in greater detail, we selected representative samples of each *Symbiodinium* taxon from each host coral species (*M. annularis*, *M. faveolata*, *M. franksi*) and made additional RFLP analyses. Different samples (from different colonies) of *Symbiodinium* A (n = 10), B (n = 12), C (n = 12), and E (n = 12) were analyzed with a panel of 12 restriction enzymes, used one at a time. These enzymes were *Dpn* II, *Taq* I, *Alw* I, *Bst*U I, *Hae* III, *Hha* I, *Hinf* I, *Mse* I, *Msp* I, *Nci* I, *Sau*96 I, and *Sty* I. Samples of *Symbiodinium* E were investigated further with the enzymes *Alu* I, *Bsp*1286 I, *Mae* III, *Mnl* I, *Sfa*N I, and *Tsp*45 I. We chose the latter enzymes based on RFLP differences among clones E^{0-1}, E^{0-2}, and E^{0-3}. All enzymes were purchased from New England Biolabs, Inc. (Beverly, MA) except for *Mae* III (Roche Diagnostics Corp., Indianapolis, IN).

Results

Identification of Symbiodinium *E*

Routine analyses of srDNAs with *Dpn* II and with *Taq* I revealed a zooxanthella in our surveys (see below) that was different from *Symbiodinium* A, B, and C (Fig. 2). We call this new RFLP genotype E (D has been assigned to a sponge symbiont [Carlos *et al.*, 1999]). Cloned genotype E srDNAs (E^{0-1}, E^{0-2}, and E^{0-3} from *Montastraea faveolata*, *M. franksi*, and *Siderastrea siderea* respectively) were more than 99% similar in sequence to one another, and more than 96% similar to srDNAs of *Symbiodinium* A, B, and C that were cloned from *M. annularis* (genotype standards A^0, B^0, and C^0). A neighbor-joining analysis of partial srDNA sequences (Fig. 3) places genotype E srDNAs within *Symbiodinium* (defined by cultured *S. microadriaticum* and *Symbiodinium* #8 [Rowan,

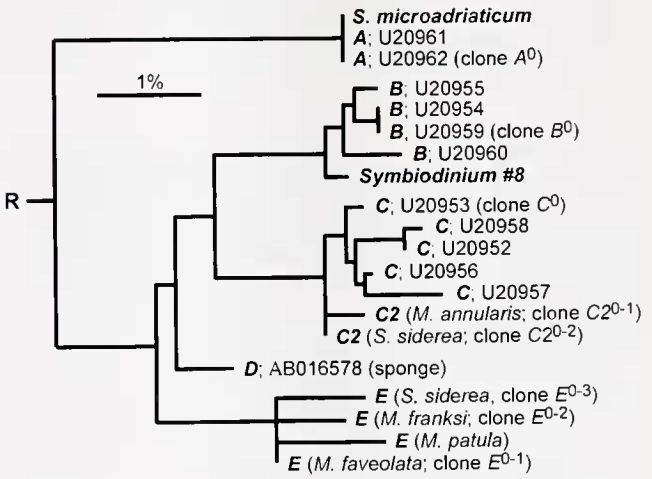

Figure 3. Inferred phylogenetic relationships among srDNAs from different zooxanthellae. Partial srDNA sequences (Rowan and Powers, 1992) were grouped by the neighbor-joining method (Saitou and Nei, 1987). *Symbiodinium microadriaticum* and *Symbiodinium* #8 are cultured zooxanthellae (Rowan and Powers, 1992). A, B, and C (followed by GenBank accession numbers) are from *Montastraea annularis* (Rowan and Knowlton, 1995); three of these correspond to standard clones A^0, B^0, and C^0 (this study). Two srDNAs labeled C2 (hosts and clone numbers in parentheses) are from Toller *et al.* (2001). D (followed by GenBank accession number) is from a sponge (Carlos *et al.*, 1999). srDNAs labeled E (host and clone numbers in parentheses) are from this study, except for that from the coral *Montipora patula*, which is from Rowan and Powers (1991a). The branch labeled R (to the left) indicates the root for this tree, obtained by including srDNA sequences from the dinoflagellates *Gymnodinium beii*, *Gyrodinium galatheanum*, *Gymnodinium simplex*, and *Polarella glacialis* (not shown).

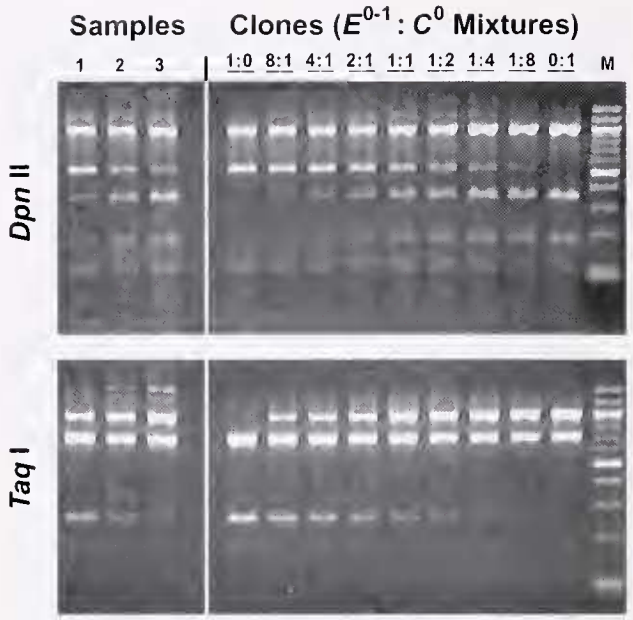

Figure 4. RFLP genotypes of mixtures of *Symbiodinium* E and C. Samples of zooxanthellae are from *Montastraea faveolata* (samples 1, 2) and *M. franksi* (sample 3); other lanes are clones E^{0-1} and C^0 singly (1:0 and 0:1, respectively) and mixed together in molar ratios ranging from 8:1 to 1:8, to obtain standards. srDNAs were amplified with host-excluding PCR primers and then digested with *Dpn* II (top panel) and with *Taq* I (bottom panel). By visual inspection, samples 1-3 contain both *Symbiodinium* E and C, in ratios of about 4:1, 1:1.5, and 1:4, respectively. Lane M contains DNA size standards, as in Figure 2.

1998]); separate from *Symbiodinium* A, B, and C; and close to a zooxanthellar srDNA from the coral *Montipora patula*, an srDNA that previously could not be assigned to either *Symbiodinium* A, B, or C (Rowan and Powers, 1991a). srDNA from *Symbiodinium* D, a dinoflagellate cultured from the sponge *Haliclona koremella* (Carlos *et al.*, 1999), is not similar to genotype E (Fig. 3). Thus, genotype E represents a distinct taxon of zooxanthella—*Symbiodinium* E.

Some samples of zooxanthellae (see below) had RFLP genotypes that implied mixtures of *Symbiodinium* E and C, based on comparisons to RFLP genotypes of synthetic mixtures of cloned genes (srDNA clones E^{0-1} and C^0; Fig. 4). As with mixtures of *Symbiodinium* A, B, or C described previously (Rowan and Knowlton, 1995; Rowan *et al.*, 1997), the apparent ratio of *Symbiodinium* E to *Symbiodinium* C in different samples varied, and did not depend on which restriction enzyme was used to differentiate these two genotypes (*e.g.*, Fig. 4, *Dpn* II digests *versus Taq* I digests).

Distribution of different taxa of Symbiodinium

At Río Cartí, *M. franksi* was observed with only two taxa of zooxanthellae—*Symbiodinium* E and C—and the same two taxa were obtained from *M. faveolata* and *M. annularis* (Fig. 5) at this reef. *Symbiodinium* E was the predominant zooxanthella from all three *Montastraea* species: it occurred in 35 of 43 corals and was the only zooxanthella detected in 18 of these. In *M. franksi* and *M. faveolata*, *Symbiodinium* E was more common in higher irradiance habitats (colonies at 1-3 m depth, tops of colonies at 3-6 m depth) than in lower irradiance habitats (colony sides at 3-6 m depth and generally below 6 m); *Symbiodinium* C exhibited the converse pattern (Fig. 5). Samples from *M. annularis* (n = 4) showed the same top and side pattern of zooxanthellar distribution within colonies (Fig. 5), although our small sample size precludes an examination across depth. A zonation pattern was often observed in comparisons of tops and sides from the 16 doubly sampled colonies that had the two types of zooxanthellae. In 12 of these colonies, the ratio of *Symbiodinium* E:C decreased from top to side, in three there was no clear difference in the ratios, and in only one colony did the ratio increase from top to side.

At Cayos Limones, *M. franksi* associated primarily with *Symbiodinium* C (Fig. 6), which was the only taxon of zooxanthella observed between 6.5 and 33 m depth (n = 53 colonies); this result is consistent with the previous study (Rowan and Knowlton, 1995) of *M. franksi* from depths

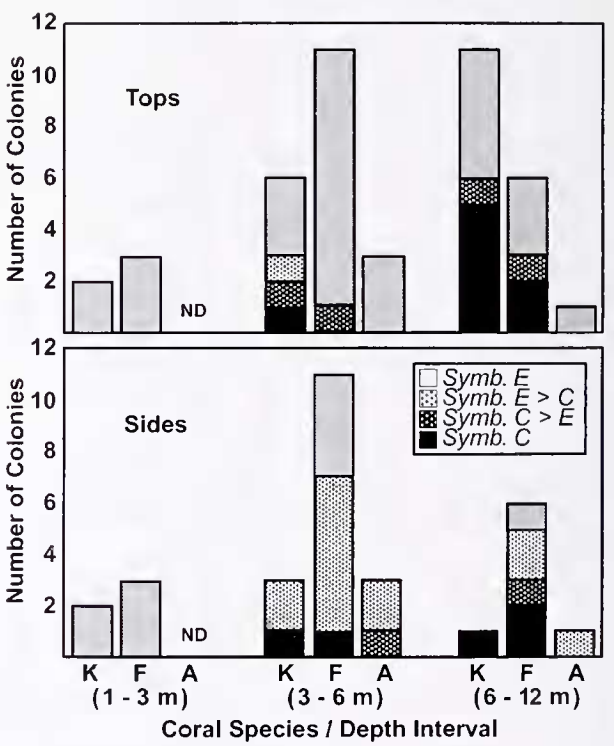

Figure 5. Occurrences of *Symbiodinium* C and E (assayed by RFLP, see Fig. 4) in colonies of *Montastraea franksi* (K), *M. faveolata* (F), and *M. annularis* (A) living in three depth intervals at Río Cartí. Top samples (upper histogram) were taken from 43 corals; 30 of these were also sampled on their sides (lower histogram). There are no data (ND) from *M. annularis* in the shallowest depth interval because no colonies were encountered there.

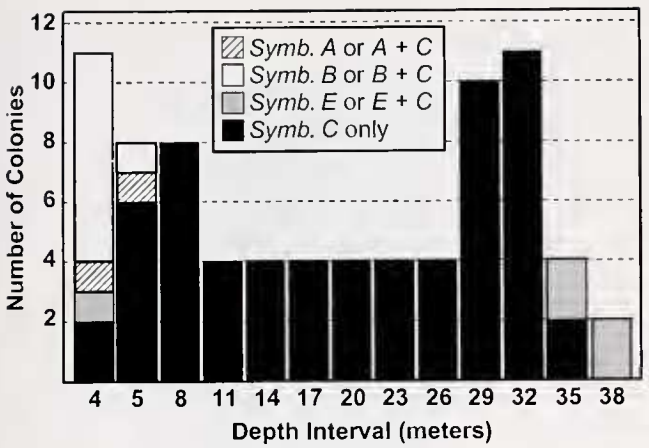

Figure 6. Occurrences of *Symbiodinium* A, B, C, and E in tops of colonies of *Montastraea franksi* living at Cayos Limones. Shallow depth intervals are 3.5-4.5 m (labeled 4) and 4.5-6 m (labeled 5); other depth intervals are 3 m wide on the centers indicated. Samples were scored as containing *Symbiodinium* A, B, C, and/or E, according to the key. More samples were analyzed at the ends of the depth range, where more than one taxon of *Symbiodinium* was observed.

between 6 and 11 m at Aguadargana, another nearby offshore reef (Fig. 1). However, in the shallowest and deepest colonies of *M. franksi*, different taxa of zooxanthellae were observed. Between 4 and 6 m, colonies contained, in order of decreasing frequency of occurrence, *Symbiodinium* B, C, A, and E. With the exception of *Symbiodinium* E in one colony, this distribution of taxa resembles that found in *M. annularis* at similar depths at Aguadargana reef (Rowan and Knowlton, 1995; Rowan *et al.*, 1997). Samples from four of the six deepest colonies of *M. franksi* (35-38 m depth) contained *Symbiodinium* E only; the other two colonies contained *Symbiodinium* C only (Fig. 6). At both the shallow and deep extremes, colonies of *M. franksi* were relatively small, encrusting forms (<0.5 m diameter).

To find out if the congeners of *M. franksi* at Cayos Limones also host *Symbiodinium* E at their lower depth limits, we sampled the deepest colonies of *M. annularis* (n = 23) and *M. faveolata* (n = 5) that we could find. They were not very deep (12-17 m and 13-15 m, respectively), and like *M. franksi* at the same depths, contained *Symbiodinium* C only (not shown).

In our limited sample of corals from two reefs at Bocas del Toro (1-15 m depth), *M. franksi* was found with *Symbiodinium* E only (1 colony), with *Symbiodinium* E and C (4 colonies), with *Symbiodinium* C only (2 colonies), or with *Symbiodinium* A only (3 colonies). *M. faveolata* was found with *Symbiodinium* C only (2 colonies) or with *Symbiodinium* A only (1 colony). The single encountered colony of *M. annularis* contained *Symbiodinium* A. We did not observe *Symbiodinium* B in any of these samples.

Other diversity in zooxanthellar srDNAs

The routine RFLP analyses (with *Dpn* II and *Taq* I) reported above indicated that all samples of zooxanthellae in this study contained srDNAs of either *Symbiodinium* A, B, C, or E, or mixtures thereof, as defined by our standard, cloned srDNA genotypes (A^0, B^0, C^0, $E^{0\text{-}1}$). However, when zooxanthellar srDNAs were analyzed in greater detail (with additional restriction enzymes; see Methods and Materials), samples of *Symbiodinium* B, C, and E (but none of 10 tested samples of *Symbiodinium* A) were found to contain additional srDNAs that could not be attributed to genotypes A^0, B^0, C^0, $E^{0\text{-}1}$, or to host srDNA. These other srDNAs appeared as additional DNA fragments in restriction digests, as described below.

Twelve selected samples of *Symbiodinium* E and clones $E^{0\text{-}1}$, $E^{0\text{-}2}$, and $E^{0\text{-}3}$ were all indistinguishable in digests with *Dpn* II (examples in Fig. 7, *Dpn* II panel) and with *Taq* I (not shown). In digests with *Mae* III, however, all of these samples had an additional DNA fragment in relatively low abundance (arrow in Fig. 7, *Mae* III panel) that was not part of the RFLP genotype of clones $E^{0\text{-}1}$ and $E^{0\text{-}2}$, but which was in the RFLP genotype of clone $E^{0\text{-}3}$. Thus, these samples apparently contained at least two srDNAs—one defined in *Mae* III digests by clones $E^{0\text{-}1}$ and $E^{0\text{-}2}$, the other by clone $E^{0\text{-}3}$. Similarly, an additional band in digests of sample srDNAs with *Mnl* I (arrow in Fig. 7, *Mnl* I panel) apparently represents the RFLP genotype of clone $E^{0\text{-}1}$ (*versus* clones $E^{0\text{-}2}$ and $E^{0\text{-}3}$). Digestion of samples with *Alu* I also yielded an additional DNA fragment (arrow in Fig. 7, *Alu* I panel), and digestion of cloned srDNAs with *Alu* I showed that the genotype of clone $E^{0\text{-}2}$ is unique. In all, additional bands like those shown in Figure 7 (arrows) were observed in 7 of 18 different restriction enzyme digestions (other digests not shown) of the 12 tested samples of *Symbiodinium* E. Therefore, srDNA in these samples of *Symbiodinium* E was clearly heterogeneous. This heterogeneity did not, however, vary qualitatively nor quantitatively among the tested samples (*e.g.*, Samples 1-3 in Fig. 7). Thus, clones $E^{0\text{-}1}$, $E^{0\text{-}2}$, and $E^{0\text{-}3}$, which are different (Fig. 7; see also Fig. 3), were obtained from indistinguishable samples of zooxanthellae.

As with *Symbiodinium* E, srDNA heterogeneity was observed in all tested samples of *Symbiodinium* B. Two digests (out of 12) revealed heterogeneity—*Hha* I and *Sty* I (examples in Fig. 8). In each of these, the additional fragments (arrows in Fig. 8) imply an srDNA with one restriction site gain relative to clone B^0. Interestingly, a cloned srDNA from *Symbiodinium* B (*Symbiodinium* #8 isolated from a Hawaiian anemone [*Aiptasia pulchella*] Rowan and Powers, 1992) has both additional sites (S8 in Fig. 8; schematic genotype on the right), suggesting that samples of *Symbiodinium* B from other hosts may also exhibit srDNA heterogeneity. In our samples of *Symbiodinium* B from *Montastraea*, within-sample srDNA heterogeneity did not vary among the 12 tested samples (*e.g.*, samples 1-4 in Fig. 8).

In the case of *Symbiodinium* C, srDNAs in all of 12 tested

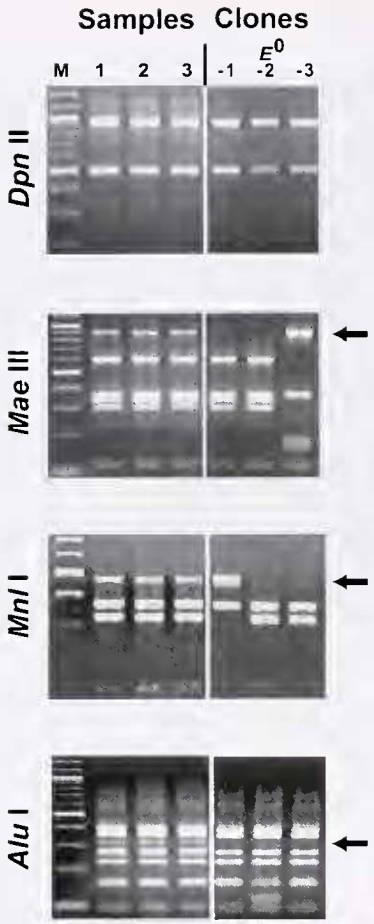

Figure 7. Examples of srDNA heterogeneity within samples of *Symbiodinium* E. srDNAs were amplified (with host-excluding PCR primers) from different samples of *Symbiodinium* E (lanes 1-3) and from srDNA clones $E^{0\text{-}1}$, $E^{0\text{-}2}$, and $E^{0\text{-}3}$ (as indicated) and then digested with *Dpn* II, *Mae* III, *Mnl* I, and *Alu* I (indicated on the left). On the right, arrows identify the positions of additional DNA fragments in lanes 1-3 that indicate srDNA heterogeneity (see text). In *Mae* III and *Mnl* I digestions, these bands were also observed in one of the three clones; for *Alu* I digestions no clone contains the indicated band. Samples are from *Montastraea franksi* (lane 1), from *M. faveolata* (lane 2), and from *M. annularis* (lane 3). Lane M contains DNA size markers as in Figure 2.

samples were also heterogeneous. However, unlike *Symbiodinium* E and B (above), within-sample srDNA heterogeneity in *Symbiodinium* C varied both qualitatively (*e.g.*, compare samples 3-5 in *Dpn* II panel, Fig. 9) and quantitatively (*e.g.*, compare Samples 1-5 in *Hinf* I panel, Fig. 9) among samples. srDNA heterogeneity was observed in as few as one or as many as six different digests (examples in Fig. 9) among the 12 samples tested. That additional variation suggested that some or all samples might have contained more than one genotype of *Symbiodinium*.

We made two analyses that might have supported this hypothesis. First, because mixtures of *Symbiodinium* A, B, C, or E vary in proportion at different locations within a coral colony (Rowan and Knowlton, 1995; Rowan *et al.*, 1997; Results), we analyzed multiple samples from colonies of *M. annularis* in which *Symbiodinium* C had been observed previously. In 14 colonies (each consisting of a cluster of columns), we sampled one column on its top and on its side; srDNA genotypes were indistinguishable in every top-*versus*-side comparison (not shown). We also sampled the tops of one or two additional columns in 13 of these colonies, and again saw no differences in zooxanthellar RFLP genotype within any colony (not shown). Second, we speculated that if the additional srDNAs did represent distinct, co-occurring zooxanthellae, their relative abundance might change under stress (*e.g.*, as in Rowan *et al.*, 1997). Transplantation of columns from deep to shallow

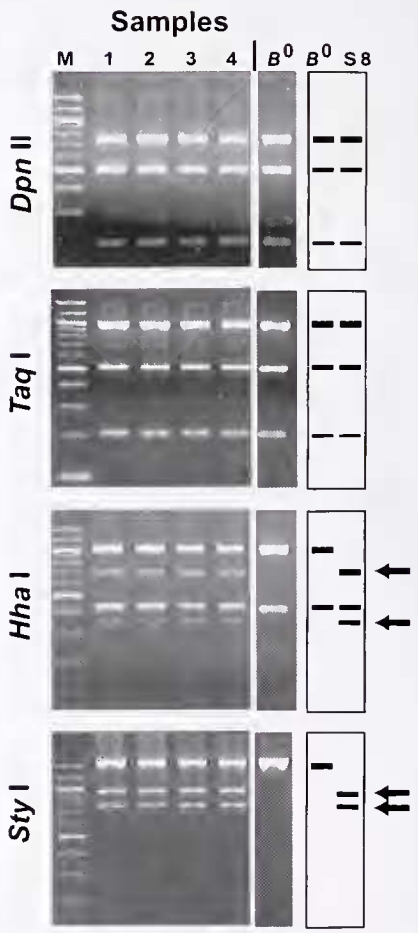

Figure 8. Examples of srDNA heterogeneity within samples of *Symbiodinium* B. srDNAs were amplified (with host-excluding PCR primers) from different samples of *Symbiodinium* B (lanes 1-4) and from srDNA clone B^0 and then digested with *Dpn* II, *Taq* I, *Hha* I, and *Sty* I (indicated on the left). On the right are schematic RFLP genotypes of clone B^0 and of an srDNA clone from *Symbiodinium* #8 (S8), obtained from its sequence (Rowan and Powers, 1992). Arrows next to the schematics identify DNA fragments that, in digests of srDNA from these samples of zooxanthellae, are additional to the DNA fragments of clone genotype B^0. Samples are from *Montastraea annularis* (lanes 1, 2), *M. faveolata* (lane 3), and *M. franksi* (lane 4). Lane M contains DNA size markers as in Figure 2.

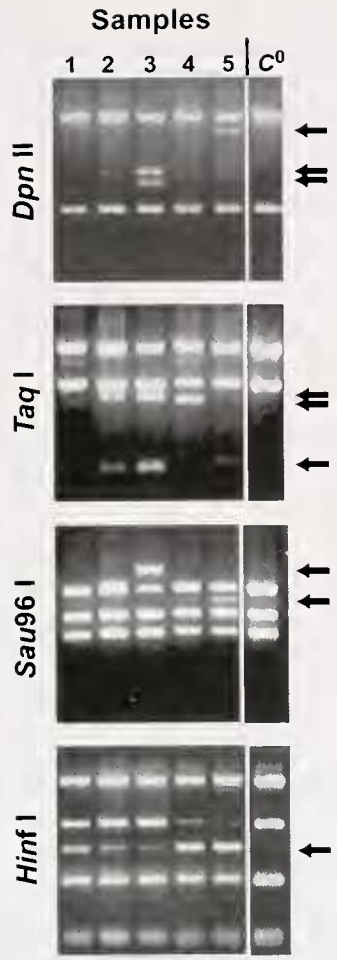

Figure 9. Examples of srDNA heterogeneity within samples of *Symbiodinium* C. srDNAs were amplified (using host-excluding PCR primers) from different samples of *Symbiodinium* C (lanes 1-5) and from srDNA clone C^0 (lane C^0) and then digested with *Dpn* II, *Taq* I, *Sau*96 I, and *Hinf* I (indicated on the left). Arrows on the right identify DNA fragments that, in digests of srDNA from these samples of zooxanthellae, are additional to the DNA fragments of clone genotype C^0. Samples are from *Montastraea annularis* (3), *M. faveolata* (1, 2, 4) and *M. franksi* (5).

habitats resulted in bleaching of all columns, and effectively reduced zooxanthellar numbers (70% reduction on average). However, neither acute stress (5 days) nor prolonged stress (*ca.* 40 days) of zooxanthellae altered the RFLP genotypes that were observed (examples in Fig. 10)—the relative abundance of distinct srDNAs had not changed compared to samples taken prior to transplantation.

Discussion

Four taxa of Symbiodinium *in the* Montastraea annularis *complex*

Previous surveys of zooxanthellar diversity in *Montastraea annularis, M. faveolata,* and *M. franksi* (Rowan and Knowlton, 1995; Rowan *et al.*, 1997) are now shown to be incomplete. In surveys of additional habitats and depths, we found (i) a fourth taxon of *Symbiodinium* (E) that was not previously reported in these corals, (ii) differences in the distribution of zooxanthellae at offshore and coastal reefs, and (iii) multiple taxa of zooxanthellae in *M. franksi,* which previously had been found to contain only *Symbiodinium* C.

Groups A, B, C, and E constitute the known diversity of coral-associated *Symbiodinium* (Rowan, 1998; this study), and *M. annularis, M. faveolata,* and *M. franksi* all associate with at least one member of each of these groups. This is a remarkable amount of taxonomic diversity—at least 12 distinct symbioses—in what was previously (Knowlton *et al.*, 1992; Rowan and Knowlton, 1995) regarded as one species of coral hosting one species of zooxanthella. Moreover, this diversity is not randomly distributed, suggesting that what was once viewed as a single quintessential generalist (Connell, 1978) is in fact a complex assemblage of ecologically more specialized entities.

Our observations from Cayos Limones now enable us to refute the speculation that *M. franksi* associates exclusively with *Symbiodinium* C—this host coral can and does form symbioses with *Symbiodinium* A, B, and E. However, at this offshore reef, the latter host-zooxanthella combinations are observed only at the margins of this coral's depth distribution (Fig. 6): shallow (*Symbiodinium* B > A > E) and very deep (*Symbiodinium* E; discussed further below). Other-

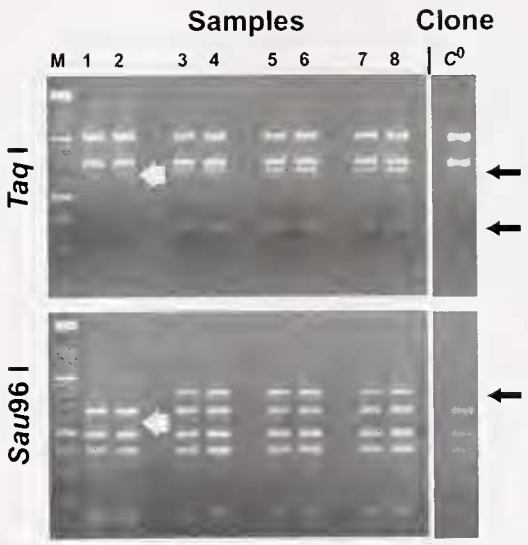

Figure 10. srDNA heterogeneity in samples of *Symbiodinium* C from four colonies of *Montastraea annularis* before and after experimental stress (see text). srDNAs were amplified (using host-excluding PCR primers) from samples of zooxanthellae (lanes 1-8) and from srDNA clone C^0 (lane C^0) and then digested with *Taq* I (top panel) and with *Sau*96 I (bottom panel). Arrows identify DNA fragments that are additional to those of genotype C^0. Samples were taken from the same colony before (lane 1) and after (lane 2) stress. Samples 3 and 4 are from another colony, before and after stress (respectively), as are samples 5 and 6 and samples 7 and 8. Lane M contains DNA fragment size standards of (top to bottom) 2642 base pairs (bp), and 1500 bp to 100 bp in 100-bp increments.

wise, *M. franksi* hosts *Symbiodinium* C throughout nearly all of its depth range (Fig. 6), where colony growth is robust. Although in shallow water the distributions of zooxanthellae (mostly *Symbiodinium* A and B) are similar in *M. franksi, M. faveolata,* and *M. annularis,* the small size of *M. franksi* colonies in shallow water may reflect a relatively poor physiological fit between this coral host and these zooxanthellae.

The main question posed by our new results is why all three species in the *Montastraea annularis* species complex at a coastal site (Río Cartí) host predominantly *Symbiodinium* E at higher irradiance (Fig. 5), instead of *Symbiodinium* A or B, as found at offshore reefs (Rowan and Knowlton, 1995; Rowan *et al.,* 1997; Fig. 6). One possible explanation is that this coastal site is characterized by environmental stress to which *Symbiodinium* E is more tolerant than are *Symbiodinium* A or B. High irradiance is a stress that may exacerbate (Brown, 1997) the many other kinds of stress found in nearshore environments (*e.g.,* fluctuations in temperature, salinity, nutrients, sediments, and underwater irradiance; see Bowden, 1983; Kirk, 1994). All of these factors can affect the stability of coral-algal symbioses (Falkowski *et al.,* 1993; Brown, 1997; Wesseling *et al.,* 1999). In the San Blas Archipelago (Fig. 1), nearshore effects associated with freshwater runoff are limited to a relatively narrow coastal band and do not reach our offshore study sites at Aguadargana and Cayos Limones (D'Croz *et al.,* 1999). *Symbiodinium* E was also common in *Montastraea* within a large coastal lagoon at Bocas del Toro, Panama (Fig. 1), an area of exceptionally high rainfall where water quality is also likely to be dominated by coastal effects.

A second (and perhaps related) question asks why *Symbiodinium* E was distributed differently at Cayos Limones, where it was common not at high irradiance but rather in the very deepest colonies of *M. franksi* (Fig. 6). Perhaps shallow and deep populations of *Symbiodinium* E are different species of zooxanthella, although we did not find any evidence to support this (see following section). Instead, we suggest that *Symbiodinium* E was actually not distributed so differently at these two sites. In both cases it was associated with marginal habitat: at great depth where *M. franksi* colonies are not large and where the reef itself disappears into sediment (Cayos Limones), and along the coast near a large river, where coral reefs are poorly developed or absent (Río Cartí). Bleaching-associated stress may be common in both habitats, due to occasional smothering by sediments in the former (*e.g.,* Wesseling *et al.,* 1999) and to near-shore conditions in the latter (see above). We propose that the *Symbiodinium* E we observed represents a taxon of zooxanthella that occurs in certain habitats not because it performs best in those habitats, but because it tolerates them, whereas *Symbiodinium* A, B, and C do not. According to this idea, *Symbiodinium* E is rare or absent from other habitats not because it performs poorly in them, but because *Symbiodinium* A, B, and C are better adapted to those habitats and somehow exclude it.

Anecdotal observations are consistent with our interpretation of *Symbiodinium* E as a stress-tolerant zooxanthella. We observed *Symbiodinium* E (diagnosed by *Dpn* II and *Taq* I digests of srDNA) in *M. faveolata* in the Bahamas (not shown), in four of seven colonies that were relatively unbleached during a natural bleaching event (D. Zawada, Scripps Institution of Oceanography, pers. comm.). We also found that *Symbiodinium* E—but not *Symbiodinium* B or C—was adept at repopulating severely bleached corals in experiments (Toller *et al.,* 2001). These experimental results suggest that, in addition to tolerating stress, *Symbiodinium* E may also be good at colonizing corals whose zooxanthellar communities have been severely disrupted by stress.

Observations of zooxanthellae related to *Symbiodinium* E in other hosts and seas imply that this taxon, like the taxa *Symbiodinium* A, B, and C (Rowan, 1998), may represent a group (clade) of zooxanthellae. Those observations include the corals *Montipora patula* in Hawaii (Rowan and Powers, 1991a; Fig. 3), *Acropora palifera* in Australia (R. R., unpubl. obs.), *Pocillopora damicornis* in the eastern Pacific (Baker, 1999), *Goniastrea aspera* in Thailand (A. Douglas, University of York, pers. comm.), and the giant clam *Hippopus hippopus* in Australia (R. R., unpubl. obs.). In the context of our hypothesis that *Symbiodinium* E is stress tolerant in *Montastraea,* it is notable that *G. aspera* occurs on reef flats—an environment that is stressful for corals, and where coral bleaching events occur regularly (Brown *et al.,* 2000). Similarly, in *P. damicornis, Symbiodinium* E was disproportionately common in unbleached colonies during an El Niño-related bleaching event (*Symbiodinium* D of Baker [1999] has an RFLP pattern that is indistinguishable from that of *Symbiodinium* E from *Montastraea* in three restriction enzyme digests; A. Baker, Wildlife Conservation Society, pers. comm.). These observations suggest that other members of the clade *Symbiodinium* E may also be stress tolerant.

The hypothesis that *Symbiodinium* E is a relatively stress-tolerant zooxanthella is based on circumstantial evidence, and should be tested in experiments in which environmental factors are controlled and physiological responses are measured. Descriptive studies of unmanipulated corals are, however, indispensable for framing realistic hypotheses in the first place.

Taxonomic interpretation of variation in zooxanthellar srDNA

We recognize the RFLP genotype E as a distinct taxon—*Symbiodinium* E—for the following reasons: (i) RFLP genotype E was common, and many samples contained only

this genotype (Fig. 5); (ii) the nonrandom distribution of RFLP genotype E (Figs. 5 and 6) strongly implies that it represents a distinct organism with distinct ecological attributes; and (iii) phylogenetic analyses of genotype E srDNAs place them within *Symbiodinium*, but distinct from srDNAs of genotypes A, B, and C (Fig. 3), which, by the same reasoning, represent distinct taxa of *Symbiodinium* (Rowan, 1998). In practice, these four taxa of *Symbiodinium* are readily identified by comparison to cloned srDNAs (RFLP genotypes A^0, B^0, C^0, and $E^{0\text{-}1}$) digested with the enzymes *Dpn* II and *Taq* I.

By analyzing zooxanthellar srDNA with additional restriction enzymes, we found that samples containing srDNA of RFLP genotype B^0, C^0, or $E^{0\text{-}1}$ also contained at least one additional srDNA of a different RFLP genotype (examples in Figs. 7-9). What do these additional srDNAs represent, taxonomically? Like an srDNA in genotype C* (Rowan and Knowlton, 1995), they appear to be from *Symbiodinium* (and not some other type of organism) because (i) they were distinguishable in fewer than one-half of different restriction digests, (ii) many of them seemed to represent simple, single restriction site changes compared to a cloned srDNA (not shown), and (iii) different srDNAs from samples of *Symbiodinium E* (Fig. 3) or of C* (Rowan and Knowlton, 1995) differed relatively little in sequence.

Do these additional srDNAs represent distinct species or strains of *Symbiodinium*? In the case of *Symbiodinium* E and B, no evidence suggests that they do. Specifically, these srDNAs were not observed by themselves, nor did they vary in relative abundance from sample to sample (Figs. 7 and 8). This contrasts with observations on srDNAs of RFLP genotypes A^0, B^0, C^0 and $E^{0\text{-}1}$, which occur alone, and also mix in a range of proportions (*e.g.*, *Symbiodinium* C and E, Fig. 4; Rowan and Knowlton, 1995; Rowan *et al.*, 1997).

Because srDNA is a multigene family in eukaryotes, srDNA heterogeneity (as seen within samples of *Symbiodinium* B and E) can reside in one organism—including dinoflagellates (Scholin *et al.*, 1993; Scholin and Anderson, 1994, 1996)—among gene-family members (Hillis and Dixon, 1991). We favor this as an explanation for our data because it is parsimonious compared to the alternative of multiple strains of zooxanthellae that for some reason always co-occur in the same relative proportion. Testing this hypothesis requires the analysis either of one dinoflagellate (*e.g.*, Yeung *et al.*, 1996) or of a clonal culture (*e.g.*, Scholin *et al.*, 1993; Rowan *et al.*, 1996).

Heterogeneity of srDNA within samples of *Symbiodinium* C was more intriguing because sample-to-sample variation was observed among colonies (Fig. 9). That observation suggested that different srDNAs within any one sample could represent different strains of *Symbiodinium*. If so, that sample-to-sample variation might also appear within one coral colony, either from place to place or time to time, especially before *versus* after an environmental change. We found no such variation (*e.g.*, Fig. 10) in corals hosting *Symbiodinium* C, which again is consistent with the hypothesis that srDNA heterogeneity is a property of individual zooxanthellae. Different patterns of srDNA heterogeneity seen among samples of *Symbiodinium C* from different corals (Fig. 9) are different zooxanthellar genotypes, but we do not know if these differences are biologically significant (*e.g.*, Scholin and Anderson, 1994, 1996).

Independent of its source, within-sample srDNA heterogeneity limits the information that can be obtained from srDNA sequences. This limitation is apparent in our analysis of *Symbiodinium* E. The sequence of clone $E^{0\text{-}1}$ implies that our RFLP analyses, using 18 enzymes (examples in Fig. 7), surveyed about 220 nucleotide positions (not shown). Heterogeneity was detected with seven enzymes, which implies a within-sample srDNA sequence diversity of about 3% (7 of 220 nucleotide positions). We do not know how this diversity is distributed; possibilities range from two srDNAs that differ at 7 of 220 positions (*ca.* 3% different srDNAs, similar to the difference between srDNAs of *Symbiodinium* A and B [Rowan and Powers, 1992]) to seven srDNAs that differ from one another at 1 of 220 positions (*ca.* 0.4% different srDNAs). Differences among srDNA clones $E^{0\text{-}1}$, $E^{0\text{-}2}$, and $E^{0\text{-}3}$ fall within this range, and there is no reason to expect any cloned srDNA to represent our samples of *Symbiodinium* E with any greater precision. Moreover, the PCR creates chimeric DNA molecules when mixed templates are amplified, and many clones obtained from those PCR products will be artifacts (Bradley and Hillis, 1997; Wintzingerode *et al.*, 1997; Darius *et al.*, 1998).

Sequences of srDNAs obtained (as clones) from *Symbiodinium* in the *M. annularis* species complex are summarized in Figure 3. Because we have evidence for only four taxa—A, B, C, and E—the multiple branches within groups B, C, and E represent sequence variation within, not among, taxa. An exception to this statement is the pair of sequences labeled $C2^{0\text{-}1}$ and $C2^{0\text{-}2}$, which came from an experimentally bleached *M. annularis* and from an unmanipulated colony of the coral *Siderastrea siderea*, respectively (see Toller *et al.*, 2001). Ecological data and RFLP analyses strongly imply that $C2^{0\text{-}1}$ and $C2^{0\text{-}2}$ represent a taxon (*Symbiodinium* C2) that is distinct from the taxon *Symbiodinium* C found commonly in unmanipulated *Montastraea* (Toller *et al.*, 2001). We stress that this taxonomic difference could not be inferred from srDNA sequence data alone, given the levels of srDNA heterogeneity within samples of *Symbiodinium* C and C2 (Toller *et al.*, 2001).

In conclusion, the problem of fully interpreting srDNA variation in natural samples of *Symbiodinium* is challenging. By themselves, srDNA sequence data contributed relatively little to understanding zooxanthellar diversity in *Montastraea*. RFLP data were much more informative, not the least because they revealed the informational limits of

srDNA sequences. Many samples of zooxanthellae from these species of coral contained more than one taxon of *Symbiodinium* (Figs. 4 and 5; Rowan *et al.*, 1997), a phenomenon that would have been difficult to understand from srDNA sequences alone. RFLP data are easily obtained, at reasonable cost, from many samples of zooxanthellae, which allows ecological data to inform taxonomic decisions.

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